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Selective Synthesis of Furfuryl Alcohol from Biomass-Derived Furfural Using Immobilized Yeast Cells

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Abstract: Furfuryl alcohol (FA) is an important building block in polymer, food, and pharmaceutical industries. In this work, we reported the biocatalytic reduction of furfural, one of the top value-added bio-based platform chemicals, to FA by immobilized *Meyerozyma guilliermondii* SC1103 cells. The biocatalytic process was optimized, and the tolerance of this yeast strain toward toxic furfural was evaluated. It was found that furfural of 200 mM could be reduced smoothly to the desired product FA with the conversion of 98% and the selectivity of >98%, while the FA yield was only approximately 81%. The gap between the substrate conversion and the product yield might partially be attributed to the substantial adsorption of the immobilization material (calcium alginate) toward the desired product, but microbial metabolism of furans (as carbon sources) made a negligible contribution to it. In addition, FA of approximately 156 mM was produced within 7 h in a scale-up reaction, along with the formation of trace 2-furoic acid (1 mM) as the byproduct. The FA productivity was up to 2.9 g/L/h, the highest value ever reported in the biocatalytic synthesis of FA. The crude FA was simply separated from the reaction mixture by organic solvent extraction, with the recovery of 90% and the purity of 88%. FA as high as 266 mM was produced by using a fed-batch strategy within 15.5 h.

Keywords: biocatalysis; bio-based platform chemicals; furans; reduction; whole cells

1. Introduction

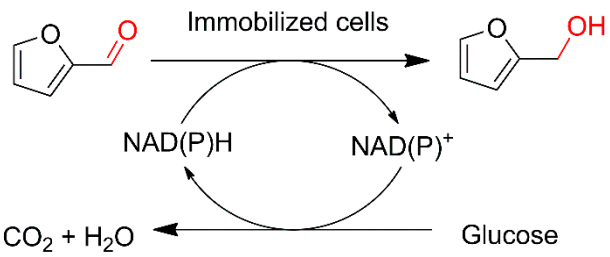
Recently, the production of biofuels and bio-based chemicals from renewable biomass has attracted outstanding interest for reducing the dependence on fossil fuel sources, as well as mitigating global warming [1,2]. Like 5-hydroxymethylfurfural (HMF), furfural that can be synthesized readily from xylose is one of the "Top 10 + 4" bio-based platform chemicals [3–5]. There exist two active functional groups, including the aromatic ring and formyl group, in furfural, which are responsible for the high chemical reactivity of this bio-based molecule. Therefore, it can be upgraded readily into a group of value-added products via some typical chemical transformations such as reduction, oxidation, and Diels-Alder reactions [6]. Furfuryl alcohol (FA) is the most important derivative of furfural, since approximately 65% of furfural produced worldwide is utilized for the synthesis of FA [4,6]. FA has been used widely in polymer, food, and pharmaceutical industries [4,6]. FA is primarily used in the production of thermostatic resins, corrosion resistant fiber glass, and polymer concrete [7]. In addition, it is a building block in the manufacture of fragrances and pharmaceuticals, and for the

synthesis of useful chemicals, such as tetrahydrofurfuryl alcohol, ethyl furfuryl ether, levulinic acid, and γ-valerolactone [6].

The industrial large-scale production of FA from furfural is performed over Cu-based catalysts, especially Cu-Cr catalysts, in gas or liquid phase [6]. Although the industrial routes have been well-established, they suffer from some problems, such as catalyst deactivation, serious environmental problems (due to the high toxicity of chromium), and overreduction of the desired product (thus leading to the formation of 2-methylfuran and furan) [5,6,8]. To address the drawbacks, chemists have devoted great efforts in the last decades. Significant advances have been achieved in the chemical catalytic hydrogenation of furfural to FA. A variety of new chemical catalysts, as well as efficient reaction engineering strategies, have also been developed to improve the production of this commercially important chemical [4,6,9].

Biotransformation is generally performed under mild conditions and is exquisitely selective, and biocatalysts are environmentally friendly [10]. Hence, biocatalysis represents a promising strategy for upgrading bio-based furans such as HMF and furfural [11], because of the inherent instability of these chemicals. Nevertheless, biocatalytic upgrading of these furans remains a great challenge, since they are proverbial inhibitors against enzymes and microorganisms [12]. Previously, many microorganisms (e.g., bacteria and yeasts) were reported to enable the detoxification of furfural into less toxic FA during the fermentation of biofuels and chemicals from lignocellulosic hydrolysates [13–18]. However, these reported microbes exhibited an unsatisfactory furan tolerance, and the biotransformation efficiencies were very low, especially at moderate to high substrate concentrations [19,20]. High substrate concentrations are highly desired for achieving satisfactory productivities in the biocatalytic synthesis of FA, which is crucial for moving these kinds of green technologies toward and into successful applications. Recently, we isolated *Meyerozyma guilliermondii* SC1103 from soil for the reduction of furans, including HMF and furfural [21]. A chemo-enzymatic method was reported for the synthesis of FA from xylose by He's group [22,23], in which the intermediate furfural derived from xylose was reduced into FA by a group of recombinant *Escherichia coli* strains. Interestingly, some *E. coli* strains exhibited good catalytic performances when the furfural concentrations were up to 200–300 mM [22,23]. Recently, Yan et al. reported the synthesis of FA using *Bacillus coagulans* NL01 [24]; the furfural tolerance of this bacterium (less than 50 mM) was unsatisfactory, although the conversion of 92% and the selectivity of 96% were obtained.

We recently performed preliminary experiments in which furfural of 50 mM was reduced into FA by resting cells of wild-type *M. guilliermondii* SC1103, with the yield of 83% and the selectivity of 96% [21]. To tap the application potential of this yeast in furfural reduction, the biocatalytic process was optimized and reaction engineering strategies were applied. Additionally, adaptively evolved yeast cells entrapped in calcium alginate were exploited for the synthesis of FA, in which NAD(P)H-dependent alcohol dehydrogenases catalyze the reduction of furfural and glucose as a co-substrate is enzymatically oxidized for generating NAD(P)H (Scheme 1). The effect of various key parameters on this reaction was studied. Interestingly, furfural of up to 200 mM could be transformed smoothly into the desired product with the selectivity of approximately 98%. The scale-up synthesis and purification of FA were carried out. In addition, a high concentration of FA was produced in the reaction mixture using a fed-batch strategy.



Scheme 1. Biocatalytic synthesis of FA from furfural with immobilized cells.

2. Results and Discussion

2.1. Process Optimization

We recently found that the catalytic activities of microbial cells were enhanced markedly when they were cultivated in the presence of a low concentration of HMF and FA (as the inducers) [25,26], likely because of the improved induction expression of the enzyme(s) responsible for the redox reactions. Therefore, the effect of the furfural addition during cell cultivation on the catalytic performances of whole cells in the synthesis of FA was examined (Figure 1a). It was found that the substrate (approximately 97%) was almost used up within 2 h in the control. FA was afforded with a yield of 89%, along with trace 2-furoic acid as the byproduct. The selectivity was up to 98%. Compared to the results in the control, no improvements in FA yields and selectivities were observed with furfural-induced cells as biocatalysts. It is well-known that furfural is more strongly inhibitory toward microbial cells than HMF and FA [27]. It can exert significantly negative effects on the cells (e.g., membrane, chromatin, and actin damage, and reduced intracellular ATP and NAD(P)H levels) during cultivation in the presence of this substance [15,27], which might offset the positive effect caused by the improved expression of the related enzymes. Therefore, no apparent improvements in the catalytic performances of these induced cells were observed compared to those of the control.

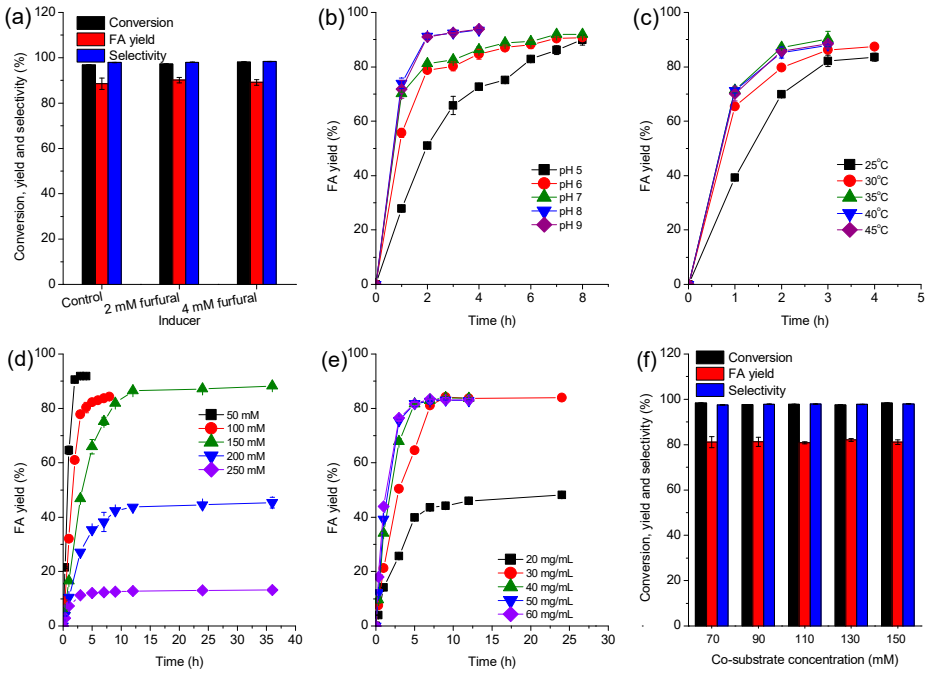


Figure 1. Effect of process parameters on the synthesis of FA: (**a**) effect of inducer; (**b**) effect of pH; (**c**) effect of reaction temperature; (**d**) effect of substrate concentrations; (**e**) effect of cell concentrations; (**f**) effect of co-substrate concentrations. General conditions unless otherwise stated: 50 mM furfural, glucose ($Mol_{glucose}$:$Mol_{furfural}$ = 3:5), immobilized cells (containing 30 mg/mL cells), 4 mL Tris-HCl buffer (100 mM, pH 8.0), 35 °C, 200 r/min; (**a**) free cells of 30 mg/mL, 2 h; (**b**) pH 5–9; (**c**) 25–45 °C; (**d**) 50–250 mM furfural; (**e**) 150 mM furfural, immobilized cells (containing 20–60 mg/mL cells); (**f**) 150 mM furfural, glucose ($Mol_{glucose}$:$Mol_{furfural}$ = 7:15–1:1), immobilized cells (containing 40 mg/mL cells), 5–7 h.

Figure 1b shows the effect of pH on the biocatalytic synthesis of FA. It was found that the catalytic activities of yeast cells were pH-dependent, and increased with the increment of buffer pH values from 5.0 to 9.0. Interestingly, FA was produced with high yields (more than 90%) and good selectivities (more than 95%) in all cases, which indicates the excellent tolerance of this strain toward acidic and alkaline environments. This is in good agreement with our recent results [21]. The subsequent studies

were performed under pH 8.0. In addition, the effect of reaction temperature on this reaction was also examined (Figure 1c). Reaction temperature exerted a significant effect on the synthetic rates of FA, whereas its influence on the maximal yields and selectivities was slight. The temperature of 35 °C was considered optimal for the biotransformation.

Then, the tolerance of the immobilized cells toward furfural was evaluated, within the concentration range of 50–250 mM (Figure 1d). Almost complete exhaustion of the substrate (>97%) occurred when its concentrations were less than 150 mM. Furthermore, the desired product FA was obtained with yields of 84–92% and selectivities of 97–99%. Nonetheless, the substrate conversions decreased remarkably to 12–49% when its concentrations were more than 200 mM. Unsatisfied FA yields (12–45%) were found, although excellent selectivities (approximately 98%) were retained. To evaluate substrate inhibition, the initial reaction rates were compared (Figure S1). The initial reaction rates increased gradually from 33 to 44 mM/h when the substrate concentrations increased from 50 to 200 mM. Additionally, a further increase in the substrate concentrations (250 mM) led to a reduced initial reaction rate (32 mM/h). This suggests that substrate inhibition toward this whole-cell biocatalyst occurs when its concentration is up to 250 mM. As mentioned above, furfural does not only severely inhibit the activities of a variety of dehydrogenases, but is also highly toxic to microorganisms. It causes cell damage, and low intracellular ATP and NAD(P)H levels [27]. In addition, substantial cell decay readily occurs in the presence of high concentrations of furfural. Hence, the moderate conversion and yield at the substrate concentration of 200 mM may be explained by these toxic effects of furfural, in spite of the absence of substrate inhibition. Great substrate toxicity plus strong substrate inhibition resulted in poor results when the furfural concentration was 250 mM.

Biocatalyst concentrations were optimized when the substrate concentration was 150 mM (Figure 1e). It was found that the FA synthesis was significantly accelerated with the increment of the cell concentrations. The reaction periods required for achieving the maximal yields decreased to 5 h when the cell concentrations were more than 40 mg/mL. Moreover, the yields and selectivities were approximately 83% and 98%, respectively. It is well-known that efficient recycling of the reduced cofactors (e.g., NAD(P)H) is crucial for efficient biocatalytic reduction, which is closely relevant to co-substrates and their concentrations. In fact, we also previously found that glucose was important for HMF reduction [21]. Consequently, the effect of the glucose concentrations on the reduction of furfural was studied (Figure 1f). As shown in Figure 1f, co-substrate concentrations had a slight impact on the biocatalytic reduction of furfural within the concentration range tested. This suggests that NAD(P)H can be effectively regenerated via oxidation of the co-substrate for furfural reduction. Therefore, the molar ratio of furfural to glucose (15:7) will be used in subsequent studies.

2.2. Improved Synthesis of FA

With optimal conditions in hand, improved synthesis of FA was conducted. Figure 2 shows time courses of the biocatalytic synthesis of FA. The maximal substrate conversion reached approximately 98% when the substrate concentration was 200 mM, which was much higher than the corresponding value (49%) at the same substrate concentration before optimization (Figure 1d). Also, a significant improvement in the substrate conversions (64% vs. 12%) was observed upon optimization when the substrate concentration was 250 mM. Unfortunately, the substrate conversions (less than 22%) remained very poor when its concentrations were more than 300 mM, indicating its significant inhibitory and toxic effects on the biocatalyst.

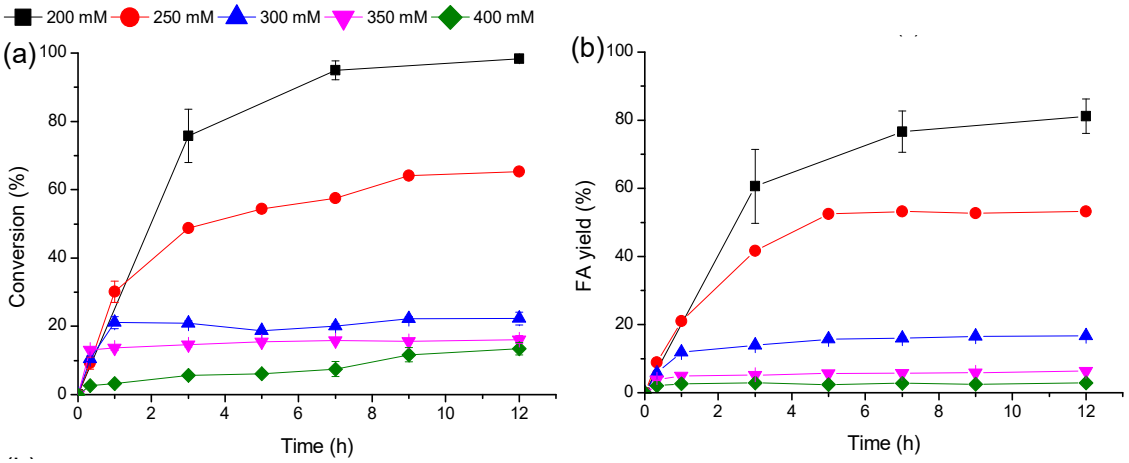


Figure 2. Time courses of the synthesis of FA from furfural: (**a**) furfural reduction; (**b**) FA synthesis. Reaction conditions: 200–400 mM furfural, glucose ($Mol_{glucose}$:$Mol_{furfural}$ = 7:15), immobilized cells (around 350 mg, containing 50 mg/mL cells), 4 mL Tris-HCl buffer (100 mM, pH 8.0), 35 °C, 200 r/min.

As shown in Figure 2b, FA was afforded with the yield of 81% after 12 h when the substrate concentration was 200 mM. In addition, only 2 mM of 2-furoic acid was produced as the byproduct. Therefore, there exists a considerable gap between the substrate conversion and the total yield of FA and byproduct (98% vs. 82%). We postulated two possible reasons for these results: (1) furans (furfural and FA) as carbon sources are metabolized by microbial cells, where 2-furoic acid as the intermediate enters into the tricarboxylic acid cycle [11]; (2) the desired product is adsorbed by the immobilized material.

To verify our assumption, biotransformation of FA and 2-furoic acid using free cells (to avoid the adsorption of the immobilized material) was carried out in the presence and absence of glucose (Figure 3). As shown in Figure 3a, FA was totally transformed in the absence of glucose after 9 h, affording 2-furoic acid with the yield of approximately 90%. A slight decrease in 2-furoic acid yields (3%) was observed with the elongation of the reaction period to 24 h. However, the oxidation of FA was greatly inhibited in the presence of glucose, since the latter was the preferred substrate for yeast cells. As shown in Figure 3a, only 20% of FA was oxidized in the presence of glucose in 12 h. Then FA conversion greatly increased to approximately 50% at 24 h, likely due to the exhaustion of glucose in the reaction mixture. Indeed, we previously found that glucose of 30 mM was quickly used up within 3 h by this yeast strain [21]. As described above, 2-furoic acid is the key intermediate in the metabolism of furfural and FA; thus, the biodegradation of this compound was examined (Figure 3b). It was found that 2-furoic acid of around 7% and 3% was degraded in the presence and absence of glucose, respectively. It suggests that this yeast strain has an extremely weak ability to biodegrade 2-furoic acid. Therefore, cellar metabolism of 2-furoic acid may make a negligible contribution to the above results.

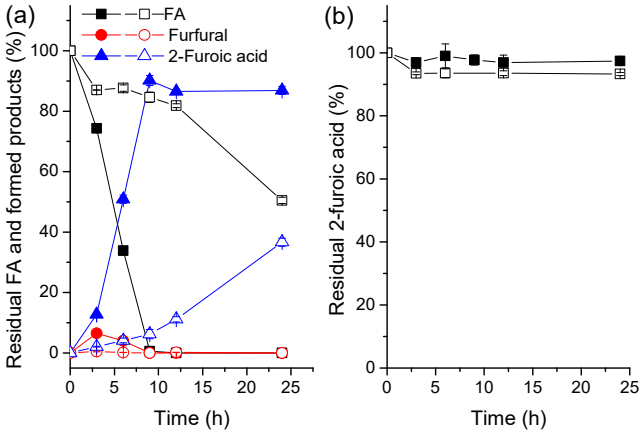


Figure 3. Transformation of FA (**a**) and 2-furoic acid (**b**) with free cells. Reaction conditions: 50 mM FA or 20 mM 2-furoic acid, in the presence (blank symbols) or absence (full symbols) of glucose (93 mM), 50 mg/mL free cells, 4 mL Tris-HCl buffer (100 mM, pH 8.0), 35 °C, 200 r/min.

Table 1 summarizes the adsorption of various immobilization materials toward the substrate and products. It was found that furfural of approximately 4% and FA of 3% were lost after incubation of 12 h in the control, likely due to evaporation, because their boiling points are approximately 170 °C. Regardless of the substances tested, much higher losses were observed in the presence of immobilization matrices compared to in the control (Table 1). In addition, losses showed a close dependence on the amounts of materials. For example, furfural of 7% and FA of 10% were reduced in the presence of 150 mg calcium alginate, whereas 350 mg of alginate beads resulted in losses of approximately 14% of furfural and FA. Compared to furfural and FA, the losses of 2-furoic acid were much lower (2–3%) in the presence of alginate beads. Therefore, the considerable adsorption of alginate beads toward the desired product FA may partially account for the lower product yields compared to the conversions.

Table 1. Adsorption of immobilization materials toward substrate and products.

Polymers (*w*/*v*)	Polymer Amount (mg)	Losses (%)		
		Furfural	FA	2-Furoic Acid
Control	none	4 ± 2	3 ± 1	n.d. [1]
Calcium alginate (2.5%)	150	7 ± 2	10 ± 1	2 ± 1
	350	14 ± 1	15 ± 3	3 ± 3
Agar (2%)	150	10 ± 1	10 ± 0	9 ± 1
	350	16 ± 1	15 ± 1	14 ± 0
Gelatin (15%)	350	26 ± 1	14 ± 0	9 ± 2
Carrageenan (3%)	350	10 ± 3	11 ± 0	15 ± 0
Poly(vinyl alcohol) (10%)	350	21 ± 1	18 ± 2	9 ± 2

Conditions: 150 or 350 mg of gel beads without the cells were incubated in 4 mL Tris-HCl buffer (100 mM, pH 8.0) at 35 °C and 200 r/min for 12 h in the presence of 200 mM furfural, 200 mM FA, or 20 mM 2-furoic acid; the changes in the concentrations of furfural, FA, and 2-furoic acid were determined, respectively. [1] none detection.

To identify promising materials that slightly adsorb the desired product for cell immobilization, a variety of commonly used polymer beads were prepared and their adsorption toward substrate and products was examined (Table 1). Unfortunately, these materials tested exhibited great adsorption capacities toward furans. The losses were dependent on the nature of the materials, as well as on the chemicals tested. In addition, most of the materials showed higher adsorption capacities toward furfural than calcium alginate (16–26% vs. 14%), with the exception of carrageenan (10%). Furthermore, considerable losses of FA (11–18%) were observed in the presence of other polymer beads. Among the polymers tested, calcium alginate showed the poorest adsorption toward 2-furoic acid, which may be an attractive material for immobilizing cells for the production of 2-furoic acid.

2.3. Scale-Up Synthesis and Separation of FA

To envisage the practicality of this bioprocess, the synthesis of FA was scaled up when the substrate concentration was 200 mM (Figure 4). As shown in Figure 4, furfural was almost used up within 5 h. The desired product FA was quickly formed in the initial stage of 5 h. FA of approximately 157 mM was finally produced, together with the formation of 2-furoic acid of around 1 mM. The yield and selectivity of FA were around 79% and 99%, respectively. The FA productivities of approximately 2.9 and 2.2 g/L/h were obtained in the scale-up synthesis, respectively, based on the FA concentrations at 5 and 7 h. After the reaction, the immobilized cells were filtered off, and the desired product was extracted three times from the reaction mixture by ethyl acetate. The crude product was furnished with the recovery of approximately 90% and the purity of 88% after evaporating the organic solvent.

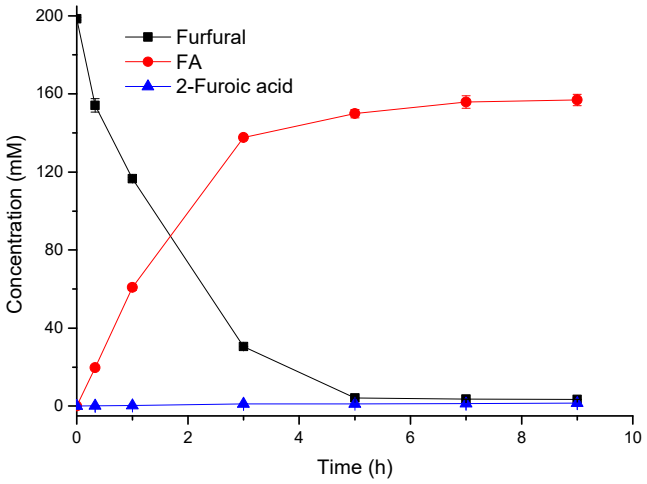


Figure 4. Scale-up synthesis of FA. Reaction conditions: 200 mM furfural, glucose ($Mol_{glucose}$:$Mol_{furfural}$ = 7:15), immobilized cells (containing 50 mg/mL cells), 20 mL Tris-HCl buffer (100 mM, pH 8.0), 35 °C, 200 r/min.

2.4. FA Synthesis by a Fed-Batch Strategy

In addition to the yield and productivity, the titer is an important index for evaluating the economic viability of a process [28], because an appropriate titer can not only significantly improve the reactor productivity, but also facilitate the product purification. Considering the high toxicity of the substrate, a fed-batch strategy was applied for accumulating the desired product of high concentrations in the reaction mixture (Figure 5). As shown in Figure 5, FA of approximately 190 mM was produced after 5.5 h. In a preliminary study, we found that the cells significantly deactivated at 5.5 h (data not shown), likely due to the great cell damage caused by toxic furfural. Accordingly, the immobilized cells were isolated from the reaction mixture, and reactivated by cultivation in fresh culture medium. It was observed that the reactivated cells were capable of transforming 90% furfural into FA within 5 h. Unfortunately, significant deactivation of the biocatalysts remained after the third substrate feeding. After 15.5 h, substrate of up to 62 mM was found in the mixture, and its concentration reduced slightly with the elongation of the reaction period. This might be ascribed to the biocatalyst deactivation caused by the toxic substrate. On the other hand, the product inhibition toward microbial cells occurred readily when its concentration was as high as 266 mM. Overall, the desired product of up to 266 mM was produced within 15.5 h, along with the formation of 2-furoic acid of 6 mM.

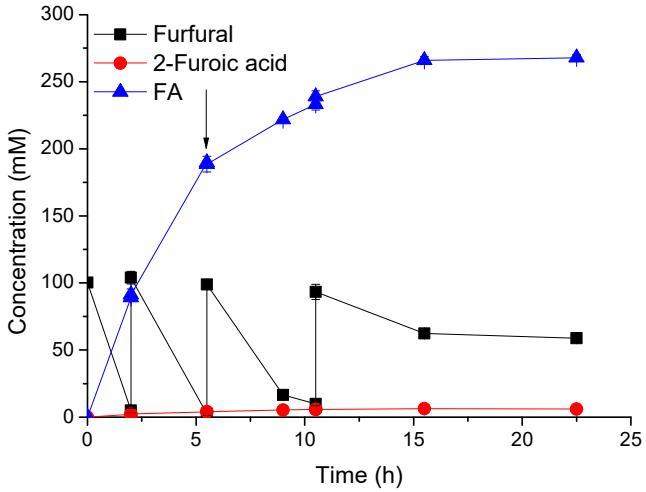


Figure 5. FA synthesis by a fed-batch strategy. Reaction conditions: 100 mM furfural, glucose ($Mol_{glucose}$:$Mol_{furfural}$ = 7:15), immobilized cells (containing 50 mg/mL cells), 8 mL Tris-HCl buffer (100 mM, pH 8.0), 35 °C, 200 r/min; after furfural was almost used up, substrate and co-substrate were supplemented. Arrows indicate the reactivation of the immobilized cells by cultivation.

To directly evidence the adsorption of immobilized material toward furans, the immobilized cells were isolated and deconstructed, followed by extraction by ethyl acetate. The furan concentrations were measured. It was found that FA of approximately 25 mM was adsorbed by the immobilized material, together with 3 mM furfural. However, 2-furoic acid was not observed. The results suggest that the desired product of at least 10% was adsorbed by the immobilized material, leading to the underestimation of the real FA yield.

Table 2 shows biocatalytic FA synthesis using a variety of microorganisms. In general, the FA productivities (<1 g/L/h) were low in the biodetoxification of lignocellulosic hydrolysates. This is due to the fact that the primary aims of these studies are not the production of FA, but the production of biofuels and chemicals from hydrolysates through fermentation. Therefore, the furfural concentrations tested were low in biodetoxification, resulting in low FA productivities. Compared to *B. coagulans* NL01 reported recently [24], *M. guilliermondii* SC1103 exhibited a much higher furfural tolerant level (42 vs. 200 mM). He et al. reported that FA was obtained in 12 h using recombinant *E. coli* CCZU-A13 cells, with yields of 94% and 74% at the furfural concentrations of 200 and 300 mM, respectively [23]. The concentrations of both the biocatalyst and co-substrate glucose were two-fold higher in He's report than those in this work, although better results (higher substrate tolerance and higher FA yields) were obtained in the former. More importantly, the productivity of FA was much higher in this work than in He's report (2.9 vs. 1.8 g/L/h).

Table 2. Comparison of FA synthesis using various biocatalysts.

Biocatalyst	Reaction Conditions	Substrate Concentration (mM)	Time (h)	C/Y [1] (%)	Selectivity (%)	Productivity [2] (g/L/h)	Ref.
Enterobacter sp. FDS8	4.6 mg (dry weight)/mL cells, pH 7, 30 °C, in lignocellulosic hydrolysate	19	3	n.a. [3]	n.a.	0.5 [4]	[20]
Clostridium acetobutylicum ATCC 824	1% of the inoculate culture, pH 5.6, 30 °C, in a sugar cane molasses medium	17	12	100 (C)	100	0.1	[15]
S. cerevisiae 307-12-F40	1% of the inoculate culture, 30 °C, in a synthetic complete medium containing 20 mM glucose	30	30	70 (Y)	n.a.	<0.1 [4]	[14]
S. cerevisiae 354	Cell concentration is not available, pH 6.7, 35 °C, in a P2 medium containing approximately 333 mM glucose	62.5	6	100 (Y)	100	1.0	[29]
B. coagulans NL01	$Mol_{glucose}$:$Mol_{furfural}$ = 5:2, 9 mg (dry weight)/mL cells, pH 7, 50 °C, in phosphate buffer	42	3	96 (C)	87	1.3 [4]	[24]
E. coli CCZU-A13	$Mol_{glucose}$:$Mol_{furfural}$ = 1:1, 100 mg (wet weight)/mL cells, pH 6.5, 30 °C, in KH_2PO_4-K_2HPO_4 buffer	200 300	12 12	94 (Y) 74 (Y)	n.a. n.a.	1.5 1.8	[23] [23]
E. coli CCZU-K14	$Mol_{glucose}$:$Mol_{furfural}$ = 3:2, 400 mM xylose, 100 mg (wet weight)/mL cells, pH 6.5, 30 °C, in KH_2PO_4-K_2HPO_4 buffer	200	24	100 (Y)	100	0.8	[22]
M. guilliermondii SC1103	$Mol_{glucose}$:$Mol_{furfural}$ = 7:15, 50 mg (wet weight)/mL cells, pH 8, 35 °C, in Tris-HCl buffer	200	7	79 (Y)	99	2.2/2.9 (5 h)	This work

[1] C, conversion; Y, yield. [2] based on the formed FA. [3] not available. [4] based on the consumed substrate.

3. Materials and Methods

3.1. Materials

M. guilliermondii SC1103 maintained in the China Center for Type Culture Collection (CCTCC, Wuhan, China; with CCTCC No. M2016144) was isolated by our laboratory [21]. Furfural (99%) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). FA (98%) was obtained from J&K Scientific Ltd. (Guangzhou, China). 2-Furoic acid (98%) was obtained from TCI (Japan). Other chemicals were of the highest purity commercially available.

3.2. Cultivation and Immobilization of Microbial Cells

Acclimatized *M. guilliermondii* SC1103 cells described in a recent report [26] were used in this work, which were cultivated prior to immobilization. Briefly, the cells were pre-cultivated at 30 °C and 200 r/min for 12 h in the yeast extract peptone dextrose medium (YPD, 1% yeast extract, 2% peptone and 2% glucose) in the presence of 0–4 mM furfural. Then, the 2% seed culture was inoculated to the fresh YPD medium in the presence of 0–4 mM furfural. After incubation at 30 °C and 200 r/min for 12 h, the cells were harvested by centrifugation (6000 r/min, 10 min, 4 °C) and washed twice with distilled water.

Cell immobilization was performed according to a recent method [26]. Typically, 2.0–6.0 g cells (cell wet weight) were mixed with 10 mL 2.5% (*w*/*v*) sodium alginate. Then, the mixture was added drop-wise from a syringe to 0.2 M $CaCl_2$ solution. The resulting gel beads (with the diameters of approximately 1.4 mm) were hardened at room temperature for 4 h. Then, the beads were washed three times with Tris-HCl buffer (100 mM, pH 7.2) and stored at 4 °C in this buffer until use. Other polymer gel beads without cells were prepared according to previous methods [30–33], with slight modifications.

3.3. General Procedure for the Synthesis of FA

Typically, 4 mL Tris-HCl buffer (100 mM, pH 8.0) containing 150 mM furfural, 70 mM glucose, and immobilized cells (containing 50 mg cells (cell wet weight) per mL buffer) was incubated at 35 °C and 200 r/min. Aliquots were withdrawn from the reaction mixtures at specified time intervals and diluted with the corresponding mobile phase prior to HPLC analysis. The initial reaction rate was calculated based on the changes in the substrate concentrations in the initial stage (usually 20 min). The conversion was defined as the ratio of the consumed substrate to the initial substrate amount (in mol). The yield was defined as the ratio of the formed FA to the initial substrate amount (in mol). The selectivity was defined as the ratio of the formed FA to the total amount of all products (in mol). All the experiments were conducted at least in duplicate, and the values were expressed as the means ± standard deviations.

3.4. Scale-Up Production and Extraction of FA

The reaction mixture was composed of 20 mL Tris-HCl buffer (100 mM, pH 8.0), 200 mM furfural, and 93 mM glucose. After the immobilized cells (containing 50 mg cells (cell wet weight) per mL buffer) were added to the reaction mixture, the reaction was conducted at 35 °C and 200 r/min. The immobilized cells were removed by filtration upon reaction. The reaction solution was saturated with NaCl by adding this substance in excess, followed by extraction three times with ethyl acetate. The organic phases were combined and dried over anhydrous Na_2SO_4 overnight. The crude product was obtained upon evaporation of the organic solvent.

3.5. FA Synthesis via Fed-Batch Feeding of Substrate

The reaction mixture containing 8 mL Tris-HCl buffer (100 mM, pH 8.0), 100 mM furfural, 47 mM glucose, and the immobilized cells (containing 50 mg cells (cell wet weight) per mL buffer) was incubated at 35 °C and 200 r/min. When furfural was almost used up, 0.8 mmol furfural and

0.38 mmol glucose were supplemented into the reaction mixture. After 5.5 h, the immobilized cells were isolated from the mixture and re-activated at 30 °C and 200 r/min for 10 h in fresh YPD medium. Upon re-activation, the immobilized cells accompanied by 0.8 mmol furfural and 0.38 mmol glucose were added into the reaction mixture. When furfural was almost used up in the reaction mixture, supplementation of furfural and glucose was repeated. The changes in the concentrations of various compounds were monitored by HPLC. After the reaction, the immobilized cells were isolated from the reaction mixture, followed by rinsing by Tris-HCl buffer (100 mM, pH 8.0) to remove furans on the bead surfaces. Then, the beads were treated ultrasonically in 3 mL sodium phosphate buffer (200 mM, pH 7.0) for 1 h, resulting in complete deconstruction of the beads. After removal of the particles by centrifugation (12,000 r/min, 10 min), the supernatant was saturated with NaCl by adding this substance in excess, followed by extraction by 6 mL ethyl acetate. The furan concentrations were determined by HPLC.

3.6. HPLC Analysis

The reaction mixtures were analyzed on an Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent Technologies, Santa Clara, CA, USA) by reversed phase HPLC equipped with a Waters 996 photodiode array detector (Waters Corporation, Milford, MA, USA). The mobile phase was the mixture of acetonitrile/0.4% $(NH_4)_2SO_4$ solution (10:90, *v*/*v*) with the flow rate of 0.6 mL/min. The retention times of 2-furoic acid, FA, and furfural were 9.1, 13.8, and 15.7 min, respectively.

4. Conclusions

In summary, an efficient biocatalytic process for FA synthesis was successfully developed using immobilized *M. guilliermondii* SC1103 cells. The yeast cells were tolerant to furfural of up to 200 mM. A high conversion (98%) and an excellent selectivity (>98%) were achieved. The desired product was significantly adsorbed by immobilization material, resulting in a relatively low yield (81%). In the scale-up synthesis, FA productivity as high as 2.9 g/L/h was obtained, which is the highest productivity of FA ever reported. FA of up to 266 mM was produced within 15.5 h by a fed-batch strategy. Although satisfactory results were obtained by this bioprocess, some problems, such as long-term stability of the biocatalyst and longer reaction periods compared to chemical methods, should be addressed in the future to move this clean technology forward to successful applications.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/9/1/70/s1, Figure S1: Time courses of substrate conversion in the reduction of furfural. The detailed methods for the preparation of other gel beads.

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